BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows in schematic form the replicase gene from Qβ and its cleavage sites as described in detail in this application as well as the location of the replicase insert in plasmid pUC18.

Figure 2 illustrates isolation and assembly of a chimeric gene containing nopaline synthase (NOS) promoter-NPTase II (NPT II) coding sequence-nopaline synthase 3'-nontranslated region. The nopaline synthase promoter was isolated on a 350-bp *Sau*3A fragment that also contained the first 44 bp of the nopaline synthase coding sequence. The sense strand of this fragment was cloned into the *Bam*HI site in M13 mp7 (Messing et al., 1981), the 44 bp was removed by using a modification of a published synthetic primer procedure (Goeddel et al., 1980) with a primer complementary to bases 22-35 of the published nopaline synthase sequence (Depicker et al., 1982), and a 308-bp promoter fragment was obtained after digestion with *Eco*RI. The flush-end of the promoter fragment was joined to a 1-kb *Bgl* II-*Bam*HI fragment carrying the NPTase II coding sequence (a *Bam*HI linker had been inserted at the *Sma* I site) at the filled-in *Bgl* II site (Beck et al., 1982). This fusion regenerates the *Bgl* II site. The chimeric gene was completed by the addition of a 260-bp *Mbo* I fragment that contained the nopaline synthase 3'-nontranslated region. This fragment, which contains a polyadenylation signal (Depicker et al., 1982), was converted to a flush-ended fragment with Klenow polymerase and cloned into the *Sma* I site of a M13 mp8 (Messing et al., 1982) to introduce *Bam*HI and *Eco*RI sites at the 5' and 3' ends, respectively. The resulting 280-bp fragment was joined to the 1,300-bp *Eco*RI-*Bam*HI nopaline synthase promoter-NPTase II coding sequence fragment to generate the complete chimeric gene.

Figure 3 illustrates structures of the pMON120 intermediate vector and chimeric gene introduced into plant cells. Plasmid pMON120 contains the following segments of DNA: the 1.7-kb pBR322*Pvu* II to *Pvu* I fragment that carries the origin of replication and *bom* site (Covarrubias et al., 1981), a 2.2-kb partial *Cla* I to *Pvu* I fragment of pTiT37 DNA that encodes an intact nopaline synthase (NOS) gene, a 2.7-kb *Cla* I-*Eco*RI fragment of Tn7 (DeGreve et al., 1981) DNA carrying the determinant for spectinomycin/streptomycin resistance, and the 1.6-kb *Hin*dIII-*Bgl* II fragment from the *Hin*dIII-18c

fragment of the pTiA6 plasmid. This T-DNA fragment is known to specify two transcripts that are not essential for tumorous growth (Willmitzer et al., 1982; Garfinkel et al., 1981). At the bottom are three chimeric genes inserted at the unique *Eco*RI site of pMON120. The chimeric nopaline synthase-NPTase II-nopaline synthase gene was inserted to give pMON129 and pMON128. In all of these examples, the first plasmid carries the inserted gene as it is drawn in the figure. The second plasmid carries the insert in the opposite orientation to that drawn. Plasmids pMON131 and pMON130 carry a chimeric nopaline synthase-NPTase I-nopaline synthase gene. The final chimeric gene is carried in plasmids pMON140 and pMON139. The bacterial NPTase II promoter and coding sequence have been joined to the nopaline synthase 3'-nontranslated region.

Figures 4A-B illustrate DNA blot hybridization analysis of *in vitro* transformants. Several hundred hormone-independent *in vitro* transformants from each experiment were pooled and total DNA was extracted (Nagao et al., 1981). The DNAs were digested with *Eco*RI and the fragments were separated by electrophoresis and transferred to nitrocellulose (Southern, 1975). (A) Hybridization with NPTase II-specific probe. A gel-purified 3.3-kb *Hin*dIII fragment from Tn*5* (Berg et al., 1975) was used as probe. Lane 1, pMON128::Ti plasmid marker; lane 2, pMON120 transformants; lane 3, pMON139 transformants; lane 4, pMON140 transformants; lane 5, pMON128 transformants; lane 6, pMON129 transformants; lane 7, pMON128 transformants; and lane 8, pMON129 transformants. Lanes 2-6 represent transformants selected for hormone-independent growth prior to scoring for kanamycin resistance; lanes 7 and 8 represent transformants selected only for kanamycin resistance on medium containing phytohormones. (B) Hybridization with NPTase I-specific probe. A gel-purified 1.2-kb *Ava* II fragment from Tn*601* (Oka et al., 1981) was used as a probe. Lane 1, pMON130::Ti plasmid marker; lane 2, pMON120 transformants; lane 3, pMON130 transformants; lane 4, pMON131 transformants; lane 5, pMON130 transformants; and lane 6, pMON131 transformants. Lanes 2-4 represent transformants selected for hormone-independent growth prior to scoring for kanamycin resistance; lanes 5

and 6 represent transformants selected only for kanamycin resistance on medium containing phytohormones.

Figure 5 illustrates growth of transformants at various antibiotic concentrations. *In vitro* transformants were obtained after cocultivation with *A. tumefaciens* strains carrying cointegrate pMON120, pMON129, or pMON131. Hormone-independent calli (1- to 2-mm diameter) from each experiment were transferred to plates (16 calli per plate) containing the antibiotic concentration shown. After 3 wk, the net growth (wet weight) at each antibiotic concentration was determined and the results were expressed as the % of control growth (growth in the absence of antibiotics). •, pMON129 transformants; Δ, pMON131 transformants; and ○, pMON120 transformants.

Figures 6A-E illustrate steps in the SEV system for plant cell transformation. The arrows represent the T-DNA border sequences. *LIH* is a region of homologous DNA for recombination. The tumor genes are represented by *tms* and *tmr* (Garfinkel et al., 1981); *OCS* and *NOS* are octopine and nopaline synthase genes, respectively. The chimeric kanamycin-resistance gene is designated as *kan*^{r}. The bacterial spectinomycin-streptomycin resistance determinant for selection of cointegrates is designated *spc/str*^{r}. Reciprocal recombination of (A) a resident Ti plasmid (pTiB6S3) and (B) pMON120 derivative (pMON128) yields (C) the cointegrate, pTiB6S3::pMON128. After cocultivation and selection for kanamycin-resistant plant cells either (D) the entire hybrid T-DNA or (E) a truncated T-DNA without tumor genes is transferred into the plant genome.

Figures 7A-B illustrate the analysis of transformed progeny. (A) Leaf callus assay. Surface-sterilized segments of leaves were placed on medium, MS salts (Gibco), B5 vitamins, 3 percent (weight to volume) sucrose, benzyl adenine (1 μg/ml), and naphthalene acetic acid (0.1 μg/ml), *p*H 5.7, containing kanamycin (100 μg/ml). Explants from wild-type plants were unable to grow on this medium, whereas explants from Horsch et al., 1984 transformed plants callused and generated shoots within 3 weeks (data not shown). The explants shown here are from four separate S_1 progeny of NPK3. One of the progeny plants is clearly sensitive to kanamycin, whereas the other three are resistant. DNA blot hybridization analysis. (B) Total plant DNA was extracted, purified by CsCl

gradient centrifugation, and digested (10 μg) with the restriction enzyme Eco RI as described (Fraley et al., 1983). After transfer of the DNA to nitrocellulose, a nick-translated DNA probe specific for transposon Tn*5* was used to identify a fragment containing the chimeric NOS/NPTII/NOS gene (Fraley et al., 1983). (Lanes a and b) Five-copy (*5c*) and one-copy (*1c*) reconstruction experiments; (lane c) DNA from wild-type (*wt*) control plants; (lane d) DNA from parental NPK3 plant (*Np3*); (lanes e to n) DNA from S_1 progeny of NPK3 plant; and (lane o) digested pMON128 plasmid showing the position of the 1.5-kb fragment of the chimeric gene. The letters *r* and *s* denote kanamycin resistance and sensitivity, respectively, in the leaf callus assay.

Please delete the paragraph appearing at page 37, line 16 through page 38, line 14.

Please insert the following paragraphs before the paragraph starting at page 38, line 15:

Infective transformation employs non-injurious infective agents of the host, such as viruses, which naturally transmit part of their genome into the host. In plants, the principal mode of transformation now being practiced is the use of the infective agent *Agrobacterium tumefaciens*. This bacterium will naturally colonize cells of any dicotyledonous plant and transmit a specific "T-region" of its Ti-plasmid into the plant chromosome. Other plant vectors useful for the transformation of plants can similarly be used.

Genes of interest can now be routinely engineered into the T-region and can be transmitted to the plant by the bacterium (see Fraley et al., 1983). Fraley et al, 1983, states as follows:

Chimeric bacterial genes conferring resistance to aminoglycoside antibiotics have been inserted into the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid and introduced into plant cells by *in vitro* transformation techniques. The chimeric genes contain the nopaline synthase 5' and 3' regulatory regions joined to the genes for neomycin phosphotransferase type I or type II. The chimeric genes were cloned into an intermediate vector, pMON120, and inserted into pTiB6S3 by recombination and then introduced into petunia and tobacco cells by cocultivating *A. tumefaciens* cells with protoplast-derived cells. Southern hybridization was used to confirm the presence of the chimeric genes

in the transformed plant tissues. Expression of the chimeric genes was determined by the ability of the transformed cells to proliferate on medium containing normally inhibitory levels of kanamycin (50 μg/ml) or other aminoglycoside antibiotics. Plant cells transformed by wild-type pTiB6S3 or derivatives carrying the bacterial neomycin phosphotransferase genes with their own promoters failed to grow under these conditions. The significance of these results for plant genetic engineering is discussed.

Abbreviations: bp, base pair(s); kb, kilobase(s); NPTase I and NPTase II, neomycin phosphotransferase, types I and II, respectively; Ti plasmid, tumor-inducing plasmid; T-DNA, transferred DNA; Ri plasmid, root-inducing plasmid.

The transformation of plant cells by virulent strains of *Agrobacterium tumefaciens* has been studied extensively by several laboratories (Chilton et al., 1977; Van Larebeke et al., 1974; Kerr et al., 1977; Braun, 1956). A small fragment of the tumor-inducing (Ti) plasmid, called transferred DNA (T-DNA), is known to be transferred to and stably incorporated in the nuclear DNA of transformed plant cells (Chilton et al., 1980; Yadav et al., 1980; Willmitzer et al., 1980). The T-DNA is actively transcribed in plant cells (Willmitzer et al., 1982; Bevan et al., 1982; Gelvin et al., 1981) and specific gene products have been shown to be responsible for the observed phytohormone-independent growth characteristics (Leemans et al., 1982; Garfinkel et al., 1981) and novel metabolic capacities (Holsters et al., 1980) exhibited by crown gall tumor cells. The transfer and insertion of T-DNA into plant DNA is thought to involve repeated nucleotide sequences present near the T-DNA "borders" (Zambryski et al., 1982; Yadav et al., 1982) as well as other genes of unknown function located in specific virulence regions outside of T-DNA (Hille et al., 1982; Klee et al., 1982).

In spite of considerable understanding of the *A. tumefaciens*-Ti plasmid system, several problems remain which limit its use as a vector for genetically modifying higher plants. Because of the high levels of phytohormones produced by crown gall tumor cells (Akiyoski et al., 1983) they have generally proven recalcitrant to attempts to induce regeneration into whole plants (Braun et al., 1976; Yang et al., 1980). Exceptions to this are cases in which, as a result of aberrant integration or spontaneous deletion events, transformed cells have lost all or part of the Ti plasmid tumor genes and can now be regenerated (Otten et al., 1981; Wullems et al., 1981a). In addition, transformation of cells by weakly virulent, mutant Ti plasmids (Barton et al., 1983) and transformation by root-inducing (Ri) plasmids (Chilton et al., 1982; White et al., 1982) have been

shown to produce callus that can be regenerated into whole plants. However, these plants often display morphological aberrations and may retain certain tumorous properties (Spano et al., 1982). Another obstacle has been the failure to obtain expression from a variety of foreign genes that have been introduced into plants (Barton et al., 1983; Chilton et al., 1981). Reasons for this include the fact that, up to now, most studies have utilized either heterologous genes from bacteria, fungi, and mammalian cells whose regulatory regions may not be recognized by the plant RNA polymerases or highly regulated plant genes which are normally expressed in specialized tissues and which may not be transcribed in undifferentiated crown gall tumor tissue.

To bypass the dependence on tumor genes for identifying transformed plant cells and to overcome the barriers to gene expression in plants, chimeric genes that function as dominant selectable markers have been assembled. These contain the neomycin phosphotransferase (NPTase) coding sequences from the bacterial transposons Tn*5* (type II) or Tn*601* (type I) joined to the 5' and 3' regulatory regions of the nopaline synthase gene from the Ti plasmid. Fraley et al., 1983, describes the construction of these chimeric genes and their introduction and expression in plant cells.

DNA Preparation. Plasmid pBR322 and its derivatives or M13 replicative form DNAs were purified by using either a Triton-X-100/CsCl procedure (Davies et al., 1980) or a large-scale alkaline lysis procedure (Ish-Horowicz et al., 1981), followed by purification on hydroxylapatite (Colman et al., 1978).

DNA fragments were isolated by electroelution into dialysis bags after polyacrylamide gel electrophoresis and band excision or by adsorption onto NA-45 DEAE membrane (Schleicher & Schuell) after agarose gel electrophoresis (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)).

The *Bam*HI synthetic DNA linkers (5' C-C-G-G-A-T-C-C-G-G, SEQ. ID. No. 1) were purchased from Collaborative Research (Waltham, MA). Other synthetic DNAs were synthesized by using a modification of the phosphite procedure (Adams et al., 1983).

Enzymes. All restriction endonucleases and the large Klenow fragment of DNA polymerase I were obtained from New England Biolabs or Bethesda Research Laboratories and were used according to the instructions of the supplier. Phage T4 DNA ligase was prepared as in Murray et al., 1979. DNA fragment assembly was carried out as described (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)).

Transformation of *Escherichia coli* cells. Plasmid DNAs were introduced into *E. coli* cells by using $CaCl_2$-treated or $RuCl_2$-treated cells (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)). The recipient *E. coli* K-12 strains were SR200 = C600 *thr pro recA56 hsdR(r^-m^+)* (Rogers et al., 1980); LE392 = ED8554 *hsdR(r^-m^+)* (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)); SR20 = GM42 = *his dam-3* (Bale et al., 1979); and the M13 phage host, JM101 (Messing et al., 1981). Cells carrying recombinant plasmids were selected or grown (or both) on Luria medium plates or broth at 37°C containing appropriate antibiotics (ampicillin, 200 μg/ml; spectinomycin, 50 μg/ml; and kanamycin, 40 μg/ml).

Introduction of pMON120 Derivatives into *A. tumefaciens*. Plasmid pMON120 or its derivatives were transferred to a chloramphenicol-resistant *A. tumefaciens* strain GV3111 = C58C1 Cm^R carrying pTiB6S3tra^c (DeGreve et al., 1981) by using a triparental plate mating procedure (Ditta et al., 1980). Briefly, 0.2 ml of a fresh overnight culture of LE392 carrying pMON120 or its derivative was mixed with 0.2 ml of an overnight culture of HB101 (Maniatis et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., p. 504 (1982)) carrying the pRK2013 (Ditta et al., 1980) plasmid and 0.2 ml of an overnight culture of GV3111 cells. The mixture of cells was spread on an LB plate and incubated for 16-24 hr at 30°C to allow plasmid transfer and recombination. The cells were resuspended in 3 ml of 10 mM $MgSO_4$ and a 0.2-ml aliquot was then spread on an LB plate containing 25 μg of chloramphenicol per ml and 100 μg each of spectinomycin and streptomycin per ml to select *A. tumefaciens* carrying the pMON120 derivatives. After incubation for 48 hr at 30°C, ≈10 colonies per plate were obtained. Control matings between HB101/pRK2013 cells and GV3111 cells never gave rise to colonies after this selection. Typically, one colony was chosen and grown at 30°C in LB medium containing chloramphenicol, spectinomycin, and streptomycin at the same concentrations given above.

Protoplast Isolation and Culture. "Mitchell" petunia plants were grown in environmental chambers under fluorescent and incandescent illumination (≈5,000 lux, 12 hr/day) at 21°C in a 50:50 mixture of vermiculite and Pro-mix BX (Premier Brands, Quebec, PQ, Canada). Leaves were surface sterilized, cut into 2-mm strips, and enzymatically digested as described (Ausubel et al., 1980). The resulting protoplasts were purified by passage through stainless steel meshes and by density floatation as described (Ausubel et al., 1980). The

protoplasts were plated in tissue culture flasks (T75, Falcon; 6 ml per flask) at a cell density of 10^5 cells per ml in culture medium, MS salts (GIBCO), B-5 vitamins, 3% (wt/vol) sucrose, 9% (wt/vol) mannitol, 1 μg of 2,4-D per ml, and 0.5 μg of benzyl adenine per ml, pH 5.7.

Cocultivation of *A. tumefaciens* Cells with Plant Protoplasts. On day 2 after protoplast isolation, aliquots (10-50 μl) of an overnight culture of *A. tumefaciens* cells were added to each flask (final bacterial cell density = 10^8 cells per ml) and cocultivation with plant cells was carried out for 24-30 hr essentially as described (Wullems et al., 1981b). On day 3, 6 ml of culture medium (lacking phytohormones and mannitol) containing carbenicillin (1.5 mg/ml) was added to each flask (final concentration = 500 μg/ml) to prevent further bacterial growth. On day 4, an additional 6 ml of the above medium (containing carbenicillin at 500 μg/ml) was added. On day 6, 0.5 ml of the cell mixture was transferred to and spread in a thin layer on the surface of double-filter feeder plates (Horsch et al., 1980). These consisted of agar medium (MS salts, B-5 vitamins, 3% sucrose, 3% mannitol, 0.1 μg of indole acetic acid per ml, and 500 μg of carbenicillin per ml at pH 5.7), a layer of *Nicotinia tabacum* suspension cells, a tight fitting 8.5-cm Whatman filter paper disc (guard disc), and a 7.0-cm Whatman filter paper disc (transfer disc). After 7-10 days, microcolonies (≈0.5 mm) were observable on the feeder plates and the transfer disc was removed and placed on selection medium (MS salts, B-5 vitamins, 3% sucrose, 500 μg of carbenicillin per ml at pH 5.7) lacking phytohormones. Within 2 wk, hormone-independent transformants could be readily distinguished as green colonies against a background of dying, brown nontransformed cells. The transformation frequency in these experiments was $\approx 10^{-1}$. The hormone-independent transformants were then transferred to medium (MS salts, B-5 vitamins, 3% sucrose, 500 μg of carbenicillin per ml at pH 7.5) containing kanamycin (50 μg/ml).

Analysis of Transformants. Octopine and nopaline synthase activities were determined as in Otten et al., 1978, with the substitution of [^{14}C]arginine (Amersham, 0.5 μCi/2.5-μl assay; 1 Ci = 3.7 x 10^{10} Bq) for the unlabeled arginine in the assay buffer. The conditions for electrophoresis were as described (Otten et al., 1978) and the resulting electrophoretograms were exposed to x-ray film (Kodak, XAR-5) for 16-24 hr. The positions of octopine, nopaline, and arginine were established by their comigration with authentic standards.

Callus for NPTase-assays were frozen in liquid N_2 and extracted by using a mortar and pestle in a minimal volume of buffer (0.2 M Tris-HCl/2 mM

EDTA/7.5% polyvinylpolypyrolidone). The crude extract was clarified by centrifugation (Eppendorf; Brinkmann) and assays were performed as described (Jimenez et al., 1980).

NPTase coding sequences were used in the initial chimeric gene constructions described in this study because plant cells were determined to be sensitive to various aminoglycoside antibiotics (unpublished data), and the expression of NPTase in yeast (Jimenez et al., 1980) and mammalian cells (Colbere-Garapin et al., 1981; Southern et al., 1982) has been previously shown to confer resistance to the antibiotic, G418. The nopaline synthase gene promoter and 3'-nontranslated regions were selected because this gene has been well characterized (Bevan et al., 1982; Depicker et al., 1982) and it is known to be expressed constitutively in most plant tissues transformed with the *A. tumefaciens* Ti plasmid (Tempé et al., 1982).

Construction of Chimeric Genes. The nopaline synthase promoter region, obtained on a 350-base-pair (bp) *Sau*3A fragment from the *Hin*dIII-23 fragment of pTiT37 (Fig. 2; Depicker et al., 1982), was engineered to remove the entire nopaline synthase coding sequence. The resulting promoter fragment that extends from base -264 to base 35 of the nopaline synthase sequence (Depicker et al., 1982) was positioned next to the *Bgl* II site located just outside the NPTase II coding sequence (Beck et al., 1982). In addition, a 260-bp *Mbo* I fragment, extending from base 1,297 to base 1,554 of the published nopaline synthase sequence (Depicker et al., 1982), was isolated from the *Hin*dIII-23 fragment. This *Mbo* I fragment contains the nopaline synthase 3'-nontranslated region and polyadenylation site. This fragment was ligated together with the *Eco*RI-*Bam*HI fragment that contained the nopaline synthase promoter and NPTase II structural gene to yield the intact chimeric gene on a 1.5-kilobase (kb) *Eco*RI fragment (Fig. 2). A second chimeric gene, containing the nopaline synthase promoter and 3'-nontranslated region joined to the NPTase I coding sequence (Fig. 3), was constructed in a similar fashion. As controls, plasmids were constructed that contained an intact NPTase II promoter and structural sequence with the nopaline synthase 3'-nontranslated region (pMON139 and pMON140; Fig. 3).

Introduction of Chimeric Genes into the Ti Plasmid. The vector pMON120 used for the transfer of the chimeric genes into *A. tumefaciens* cells is shown in Fig. 3. Its essential features include (*i*) a segment of pBR322 DNA for replication in *E. coli*, (*ii*) a segment from pTiT37 that contains a functional nopaline synthase gene to facilitate the rapid identification of transformants, (*iii*) a segment of Tn7 carrying the spectinomycin/streptomycin-resistance

determinant for selection in *A. tumefaciens*, (*iv*) a DNA segment obtained from the pTiA6 T-DNA fragment *Hin*dIII-18c (see T-DNA map, Leemans et al., 1982), which is included to provide homology for recombination with a resident octopine-type Ti plasmid in *A. tumefaciens*, and (*v*) unique restriction sites (*Eco*RI and *Hin*dIII) for insertion of the chimeric genes. The pMON120 plasmid and derivatives were introduced into *A. tumefaciens* as described above.

Selection of Kanamycin-Resistant Petunia Transformants. Several hundred hormone-independent calli (1-2 mm in diameter) obtained from cocultivation experiments with *A. tumefaciens* strains carrying pTiB6S3::pMON120 (or derivatives) recombinant plasmids were pooled and analyzed by DNA blot hybridization for the presence of the chimeric genes (Fig. 4). The results confirm the presence of the expected 1.6-kb *Eco*RI fragment, which carries the chimeric nopaline synthase-NPTase II-nopaline synthase gene in pMON128 and pMON129 transformants, and the control NPTase II-NPTase II-nopaline synthase construct in pMON139 and pMON140 transformants (Fig. 4A).

Similar results were obtained for pMON130 and pMON131 transformants, which contain the chimeric nopaline synthase-NPTase I-nopaline synthase gene on a 1.5-kb *Eco*RI fragment (Fig. 4B). No hybridization with either the Tn*5*- or Tn*601*-specific probe was detected in transformants containing only the pMON120 vector. Other minor brands of hybridization are present in the pMON129 and pMON140 transformants; these may be attributable to partial digestion or aberrant integration events and their assignment awaits further analysis of clonal tissue. Blot hybridization analysis of DNA from these transformants using T-DNA-specific probes confirmed the presence of the expected internal T-DNA fragments in the transformed tissues and ruled out any possibility that the plant tissue was contaminated by *A. tumefaciens* cells (data not shown).

Other transformed, hormone-independent calli from these experiments were transferred to agar medium containing kanamycin (50 μg/ml) and these were scored after 2-3 wk for resistance to the antibiotic. All transformants obtained from experiments utilizing pMON120, pMON139, or pMON140 failed to grow on medium supplemented with kanamycin, whereas all the transformants from experiments utilizing pMON128, pMON129, pMON130, or pMON131 grew on medium containing the antibiotic at rates comparable to growth on normal medium. A quantitative assessment of the level of resistance conferred by the chimeric genes is shown for pMON120, pMON129, and pMON131 (Fig. 5). The results are based on the net growth of independent transformants on medium containing the levels of antibiotic shown in the figure, compared to

growth in the absence of antibiotics. It is apparent that transformants containing the chimeric nopaline synthase-NPTase II-nopaline synthase gene (pMON129) require ≈20-fold higher levels of kanamycin to depress net growth by 50% in comparison to transformants lacking the chimeric gene (pMON120). Similar results were obtained for pMON128, which contains the chimeric gene in the opposite orientation in the pMON120 vector (not shown). Transformants containing pMON139 and pMON140 have dose responses identical to pMON120. Transformants containing pMON130 or pMON131 (chimeric nopaline synthase-NPTase I-nopaline synthase gene) are less resistant to kanamycin than those containing pMON128 or pMON129 (results shown for pMON130). However, this level of resistance (≈3-fold greater than control cells) is still quite adequate for selection (see below).

Additional cocultivation experiments were carried out without hormone-independent selection (i.e., medium supplemented with phytohormones which support the growth of nontransformed cells). The resulting microcolonies (≈1 mm) were transferred to phytohormone-supplemented medium containing kanamycin (50 μg/ml) and within 2-3 wk, growing colonies were readily observable on plates containing cells that were transformed with pMON128, pMON129, pMON130, or pMON131. The frequency of transformation obtained by using antibiotic selection was comparable to that obtained by using hormone-independent selection. Opine (data not shown) and Southern hybridization analysis (Fig. 4A, lanes 7 and 8; Fig. 4B, lanes 5 and 6) of the kanamycin-resistant colonies confirmed that they were indeed transformants. No growing colonies were observable on plates containing cells transformed by pMON120, pMON139, or pMON140 plasmids.

The expression of the prokaryotic NPTase I and NPTase II enzymes in plant cells by using the intermediate vector pMON120 probably depends on transcription from the nopaline synthase promoter. Support for this comes from the facts that (*i*) the prokaryotic genes with their own promoters do not confer antibiotic resistance to petunia cells (Figs. 3 and 5) and (*ii*) all of the constructions function identically in either orientation in the pMON120 vector, suggesting that transcription does not initiate elsewhere in the vector. RNA blot hybridization experiments have confirmed the presence of NPTase II-specific mRNA in the transformed tissues and nuclease S1 mapping experiments demonstrate the expected 5' and 3' ends for the chimeric NPTase II mRNA (data not shown). In addition, low levels of neomycin-dependent NPTase II activity have been reproducibly observed in crude cell extracts from tissues transformed with pMON128 or pMON129 (no activity has been detected in

extracts from control cells or cells transformed with pMON120, pMON139, or pMON140).

The useful range of these chimeric antibiotic resistance genes appears to be quite broad. In addition to the results presented for petunia, successful selection of aminoglycoside-resistant transformants has also been demonstrated for tobacco, sunflower, and carrot (results not shown). It seems likely that most plants within the host range of *A. tumefaciens* could be transformed and identified in this manner. Those plant cells that are not particularly sensitive to kanamycin may be killed by other aminoglycoside antibiotics. In this respect pMON128 (or pMON129) and pMON130 (or pMON131) also function to confer resistance to G418 and neomycin on petunia, carrot, sunflower, and tobacco (unpublished data).

The availability of dominant selectable markers on small plasmids such as pMON120 should facilitate the development of alternate, non-*A. tumefaciens*-mediated methods for transforming plant cells such as spheroplast fusion (Hasezawa et al., 1981) or the use of liposomes (Fraley et al., 1982) or calcium-phosphate (Krens et al., 1982) techniques. These chimeric genes should also prove useful as markers in somatic hybridization experiments or as sensitive probes for studying promoter function. Finally, two obvious but significant aspects of the results presented in Fraley et al., 1983, are (*i*) it should now be possible, by using Ti plasmids that have the tumor genes (i.e., tms and tmr loci, Garfinkel et al., 1981) deleted, to obtain kanamycin-resistant transformants that can be readily and reproducibly regenerated into phenotypically normal plants, and (*ii*) there is no reason to believe that NPTase I and NPTase II are unique in their ability to be expressed in plant cells and it is quite likely that other bacterial, fungal, or mammalian genes, including those whose products could be expected to modify plant properties in a useful manner, could also be successfully engineered and expressed.

Simple coincubation (growing plant cells and bacterial cells together) has been shown to be extremely effective in transforming plant protoplasts and leaf disks, and whole transformed plants have now been regenerated in numerous plant species (see Horsch et al., 1984). Horsch et al., 1984, states as follows:

Morphologically normal plants were regenerated from *Nicotiana plumbaginifolia* cells transformed with an *Agrobacterium tumefaciens* strain containing a tumor-inducing plasmid with a chimeric gene for kanamycin resistance. The presence of the chimeric gene in regenerated plants was demonstrated by Southern hybridization analysis, and its expression in plant tissues was confirmed by the ability of leaf segments to form callus on media

containing kanamycin at concentrations that were normally inhibitory. Progeny derived from several transformed plants inherited the foreign gene in a Mendelian manner.

Agrobacterium tumefaciens, the causative agent of crown gall disease, is capable of transferring a DNA segment (designated T-DNA), located between specific border sequences, from its tumor-inducing plasmid (Ti plasmid) into the nuclear DNA of infected plant cells (Chilton et al., 1977; Van Larebeke et al., 1974; Chilton et al., 1980; Yadav et al., 1980; Willmitzer et al., 1980). Expression of T-DNA-encoded tumor genes in the transformed cell provides a selectable trait for recognition of those cells in culture; namely, the ability to grow on medium without added phytohormones. Unfortunately, this trait interferes with regeneration of normal fertile transformed plants (Braun, 1956; Braun et al., 1976; Yang et al., 1980).

Recently, Fraley et al, 1983, and others (Herrera-Estrella et al., 1983) have constructed chimeric genes that function as dominant selectable markers in plant cells, thus making the tumor genes unnecessary for identification of transformants. The chimeric gene of Fraley et al., 1983, and Horsch et al., 1984, contains the coding sequence of the bacterial gene for neomycin phosphotransferase II (NPTII) joined to the 5' and 3' regulatory regions of the nopaline synthase (NOS) gene, which is expressed constitutively in higher plant cells (Tempé et al., 1982). Fraley et al., 1983, has shown that petunia and tobacco cells transformed with this chimeric NOS/NP-TII/NOS gene are readily selected and are highly resistant to kanamycin.

Horsch et al., 1984, now report that kanamycin-resistant plant cells obtained with their vector system regenerate to morphologically normal plants. These plants carry a functional kanamycin-resistance gene and produce viable seeds. Analysis of progeny shows that the chimeric kanamycin-resistance gene is inherited and is expressed as a dominant Mendelian trait.

Horsch et al., 1984, used the previously described pMON120 intermediate vector to introduce the chimeric NOS/NPTII/NOS gene into the *A. tumefaciens* Ti plasmid. The pMON120 plasmid also contains an intact NOS gene as a scorable transformation marker. This NOS fragment includes the nopaline-type T-DNA right border sequence (Zambryski et al., 1982; Yadav et al., 1982). Because this additional border sequence is initially carried on a separate plasmid, Horsch et al., 1984, refers to their system as the split end vector (SEV) system. Figure 6 shows how this system is used. The pMON128 plasmid, pMON120 containing the chimeric kanamycin-resistance gene (Fig. 6B), is introduced by conjugation into *A. tumefaciens* cells, where homologous

recombination with a resident octopine-type Ti plasmid, pTiB6S3 (Fig. 6A), occurs. The resultant cointegrate plasmid pTiB6S3::pMON128 (Fig. 6C) contains a hybrid T-DNA in which the nopaline-type right border sequence is positioned between the kanamycin-resistance gene and the tumor genes of the resident Ti plasmid. Use of the nopaline T-DNA border sequence during infection results in the transfer of a short T-DNA segment (Fig. 6E) which contains the kanamycin-resistance gene and an intact NOS gene but does not contain genes for tumor formation or octopine synthase. The short-transfer transformants can be regenerated to give intact plants as described below.

Transformation of *Nicotiana plumbaginifolia* cells was carried out with the engineered *A. tumefaciens* train containing the chimeric NOS/NPTII/NOS gene by the method of cocultivation (Fraley et al., 1983). Leaf mesophyll protoplasts of *N. plumbaginifolia* were obtained and cultured as described by Pollock et al., 1983. Two-day-old cultures of protoplast-derived cells were inoculated with live *A. tumefaciens* cells (10^7 cells per milliliter). After 48 hours of coculture, the plant cells were collected by centrifugation (100*g* for 5 minutes), washed twice, and replated in medium containing carbenicillin (500 μg/ml) to kill the remaining bacteria. On the sixth day after isolation of protoplasts, the procedure described for petunia cells (Fraley et al., 1983) was used to transfer the cultures from liquid to feeder plates (Horsch et al., 1980) of the same medium. After 7 to 10 days on the feeder plates, the colonies were transferred to fresh medium (without feeder cells). After another 7 days, colonies were transferred to a different medium, MS salts (Gibco), B5 vitamins, 3 percent sucrose, carbenicillin (500 μg/ml), kanamycin (100 μg/ml), benzyl adenine (1.0 μg/ml), and naphthalene acetic acid (0.1 μg/ml); *p*H 5.7. After 7 days, the most promising colonies (largest and greenest) were picked from the filter paper substrate and transferred to the same medium at low density, 10 colonies per petri plate (100 by 15 mm).

In the first experiment of Horsch et al., 1984, four kanamycin-resistant colonies were recovered from approximately 8×10^4 protoplasts cocultivated with pTiB6S3::pMON128. No resistant colonies were found among the same number of untreated control colonies. Southern blot hybridization with an NPTII specific probe showed that all four resistant colonies contained the chimeric gene (data not presented). One of the four colonies was morphogenic and produced a kanamycin-resistant plant, NPK3.

Leaf segments from NPK3 were able to form callus and proliferate new shoots on medium containing kanamycin (100 μg/ml) (Fig. 7A). In contrast, leaf segments from wild-type plants were completely inhibited. Analysis with

Southern blot hybridization showed that the 1.5-kilobase (kb) Eco RI fragment containing the chimeric NOS/NPTII/NOS gene was present in the leaves of NPK3 but not in tissue from wild-type plants (lanes c and d in Fig. 7B).

Twenty-one first-generation progeny plants (S_1) from the self-fertilized transformed parent NPK3 were grown to maturity and tested for kanamycin resistance in the leaf callus assay. Fifteen of the 21 were able to form callus on medium with kanamycin (100 μg/ml). Another 80 seedlings (germinated under sterile conditions) were transferred to medium containing kanamycin (100 μg/ml). Of these, 62 grew several times larger and formed callus, whereas 18 ceased growth and did not form callus. Thus the trait was inherited in a Mendelian manner with a 3:1 ratio. The final proof of the correspondence between the presence of the chimeric gene and the antibiotic-resistant phenotype was established by the perfect correlation between inheritance of the chimeric gene and the kanamycin-resistant phenotype in S_1 plants (lanes e to n in Fig. 7B).

Three subsequent cocultivation experiments gave high frequencies of transformation, averaging 6 percent of the total colonies or 1.2 percent of the total initial protoplasts plated. The control populations consistently failed to yield any kanamycin-resistant colonies. Most (about 90 percent) of the kanamycin-resistant colonies produced both octopine and nopaline and were nonmorphogenic, as expected for transformants arising when the octopine T-DNA right border was utilized (Fig. 6D). About 10 percent of the colonies were morphogenic, producing shoots that could be excised, rooted, and grown in soil. Of 22 plants examined, 9 were escapes or revertants that showed none of the markers of transformation. The other 13 plants produced nopaline, but not octopine, and were resistant to kanamycin as measured by a leaf callus-induction assay.

The S_1 progeny from three of the independently isolated, nopaline-producing and kanamycin-resistant plants (NPK7, NPK9, NPK10) were scored for nopaline content. In each case, the progeny showed normal Mendelian inheritance and expression of the inserted DNA segment: 71 of 105 progeny of NPK7, 39 of 48 progeny of NPK9, and 34 of 44 progeny of NPK10 produced nopaline. In addition, axenically grown seedings from each of the transformants showed similar segregation for ability to form callus on medium containing kanamycin (100 μg/ml). For example, 37 of 51 progeny of NPK10 formed callus in the presence of kanamycin, and all 37 resistant progeny produced nopaline.

Horsch et al., 1984, has shown that (i) the chimeric NOS/NPTII/NOS gene is expressed in *N. plumbaginifolia*, (ii) the regenerated transformed plants are phenotypically normal and fertile, and (iii) normal Mendelian inheritance of an engineered gene can occur in the progeny of transformed plants. The genetic transmission of chimeric antibiotic-resistance genes has now been confirmed for the S_2 progeny of the NPK3 plant.

Normal Mendelian inheritance of the chimeric gene has also been demonstrated for petunia plants transformed with pMON120-type vectors. The availability of dominant selectable markers and transformation vectors that permit the regeneration of phenotypically normal plants will greatly facilitate studies of gene expression and regulation in plants.

In mammals, naturally infective retroviruses have been used to construct naturally transforming vectors which insert engineered DNA into the mammalian chromosome, in a manner similar to *Agrobacterium tumefaciens*. This transformation mechanism is considered extremely promising for animal and human gene therapy (see Anderson, 1984).

Please delete the paragraph appearing on page 46, line 25 through page 47, line 11.

Please insert at page 47, before the paragraph starting on line 12, the following paragraphs:

All of the patents and other publications cited in this specification are indicative of the level of skill and knowledge of those skilled in the arts to which the present invention pertains. All publications, whether patents or otherwise, referred to previously or later in this specification are herein separately incorporated by reference except as noted below. Although full incorporation of the individual publications is intended, it is recognized that those of ordinary skill in the art can readily determine from the incorporated publications those sections which are most relevant to the present invention and those sections which could be deleted without loss of understanding.

Publications specifically excluded from incorporation by reference into this specification are as follows:

Adams, S.P. et al., *J. Am. Chem. Soc.*, 105:661-663 (1983).

Akiyoski, D. et al., *Proc. Natl. Acad. Sci. USA*, 80:407-411 (1983).

Ausubel, F. et al., *Plant Mol. Newsl.*, 1:26-32 (1980).

Bale, A. et al., *Mutat. Res.*, 59:157-165 (1979).
Barton, K. et al., *Cell*, 32:1033-1043 (1983).
Beck, E. et al., *Gene*, 19:327-336 (1982).
Berg, D. et al., *Proc. Natl. Acad. Sci. USA*, 76:3628-3632 (1975).
Bevan, M. et al., *J. Mol. Appl. Genet.*, 1:539-546 (1982).
Braun, A., *Cancer Res.*, 16:53-56 (1956).
Braun, A. et al., *Proc. Natl. Acad. Sci. USA*, 73:496-500 (1976).
Chilton, M.D. et al., *Cell*, 11:263-271 (1977).
Chilton, M.D. et al., *Proc. Natl. Acad. Sci. USA*, 77:4060-4064 (1980).
Chilton, M.D. et al., *Stadler Genet. Symp.*, 13:39-51 (1981).
Chilton, M.D. et al., *Nature (London)*, 295:432-434 (1982).
Colbere-Garapin, F. et al., *J. Mol. Biol.*, 150:1-14 (1981).
Colman, A. et al., *Eur. J. Biochem.*, 91:303-310 (1978).
Covarrubias, L. et al., *Gene*, 13:25-35 (1981).
Davies, R.W. et al., *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), p. 116 (1980).
DeGreve, H. et al., *Plasmid*, 6:235-248 (1981).
Depicker, A. et al., *J. Mol. Appl. Genet.*, 1:561-574 (1982).
Ditta, G. et al., *Proc. Natl. Acad. Sci. USA*, 77:7347-7351 (1980).
Fraley, R. et al., *Current Topics in Microbiology and Immunology*, eds. Hofschneider, P. & Goebel, W. (Springer, New York), 171-192 (1982).
Garfinkel, D. et al., *Cell*, 27:143-155 (1981).
Gelvin, S. et al., *Plasmid*, 6:17-29 (1981).
Goeddel, D. et al., *Nucleic Acids Res.*, 8:4057-4074 (1980).
Hasezawa, S. et al., *Mol. Gen. Genet.*, 182:206-210 (1981).
Herrera-Estrella et al., *EMBO J.* 2:987 (1983).
Hille, J. et al., *Plasmid*, 7:107-118 (1982).
Holsters, M. et al., *Plasmid*, 3:212-230 (1980).
Horsch, R. et al., *In Vitro*, 16:103-108 (1980).
Ish-Horowicz, D. et al., *Nucleic Acids Res.*, 9:2989-2998 (1981).
Jimenez, A. et al., *Nature (London)*, 287:869-871 (1980).
Kerr, A. et al., *Nature (London)*, 265:560-561 (1977).
Klee, H. et al., *Bacteriol*, 150:327-331 (1982).
Leemans, J. et al., *EMBO J.*, 1:147-152 (1982).
Messing, J. et al., *Nucleic Acids Res.*, 9:309-321 (1981).
Messing, J. et al., *Gene*, 19:269-276 (1982).
Murray, N.E. et al., *J. Mol. Biol.*, 132:493-505 (1979).
Nagao, R. et al., *DNA*, 1:1-9 (1981).

Oka, A. et al., *J. Mol. Biol.*, 147:217-226 (1981).

Otten, L. et al., *Biochim. Biophys. Acta*, 527:497-500 (1978).

Otten, L. et al., *Mol. Gen. Genet.*, 183:209-213 (1981).

Pollock, K. et al., *Plant Cell Rep.* 2:36 (1983).

Rogers, S.G. et al., *Gene*, 11:187-195 (1980).

Southern, E., *J. Mol. Biol.*, 98:503-517 (1975).

Southern, P. et al., *J. Mol. Appl. Genet.*, 1:327-341 (1982).

Spano, L. et al., *Pflanzenphysiol.*, 106:87-92 (1982).

Tempé, J. et al., *Molecular Biology of Plant Tumors*, eds. Kahl, G. & Schell, J. (Academic, New York), 427-449 (1982).

Van Larebeke, N. et al., *Nature (London)*, 252:169-170 (1974).

White, F. et al., *Proc. Natl. Acad. Sci. USA*, 79:3193-3198 (1982).

Willmitzer, L. et al., *Nature (London)*, 287:359-361 (1980).

Willmitzer, L. et al., *EMBO J.*, 1:139-146 (1982).

Wullems, G. et al., *Cell*, 24:719-727 (1981a).

Wullems, G. et al., *Proc. Natl. Acad. Sci. USA*, 78:4344-4348 (1981b).

Yadav, N. et al., *Nature (London)*, 287:458-461 (1980).

Yadav, N. et al., *Proc. Natl. Acad. Sci. USA*, 79:6322-6326 (1982).

Yang, F. et al., *Mol. Gen. Genet.*, 177:707-714 (1980).

Zambryski, P. et al., *J. Mol. Appl. Genet.*, 1:361-370 (1982).